United States Patent Application

of

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for

Compositions, Methods, and Kits for Isolating Nucleic Acids Using

Surfactants and Proteases

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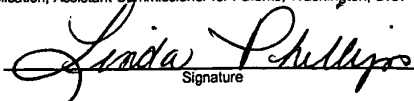
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Compositions, Methods, and Kits for Isolating Nucleic

Acids Using Surfactants and Proteases

Field of the Invention

5   The invention relates to compositions and methods for releasing and for isolating

nucleic acids from biological samples, preferably from whole tissue. The invention also

provides kits for isolating and/or releasing nucleic acids from biological samples.

Background of the Invention

Current methods for isolating nucleic acids from cultured cells generally include

10   lysing the cells and inactivating nucleases using chaotropic salts, such as guanidine

hydrochloride or guanidinium thiocyanate, and a nonionic surfactant. The released

nucleic acids are then selectively precipitated from solution. The combination of

chaotropic salts and nonionic surfactants, however, typically are ineffective at

penetrating whole tissues to release nucleic acid.

15   When cells are lysed to release nucleic acids, endogenous nucleases, enzymes

that degrade nucleic acids (DNA and RNA), are also released. Thus, when minimally

degraded (i.e., high integrity) nucleic acid is desired, one tries to minimize nuclease

activity as much as possible. Deoxyribonucleases (DNAses), which are nucleases that

degrade DNA, can often be inactivated by adding divalent cation chelators to the

20 . reaction compositions. Free magnesium ions, which are typically needed for DNAse

activity, are bound or complexed by the addition of chelators. Thus, chelators typically

diminish or inactivate endogenous DNAses. Many endogenous ribonucleases

(RNAses), nucleases that degrade RNA, however, do not typically need divalent

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cations, such as magnesium, for their activity. Such RNAses are unaffected by the addition of chelators. To inactivate RNAses during RNA isolation procedures, therefore, one may add to the reaction composition inhibitors of RNAse activity.

Various chemical disruption methods, using surfactants, chaotropes, proteases, bile salts, organic solvents, and harsh acidic or basic conditions have been employed to lyse cells and release nucleic acid. Proteinase K, a broad specificity protease, has also been employed. To increase the activity of Proteinase K, denaturing agents, such as anionic detergents and chaotropes have been used in combination with Proteinase K. The nucleic acid obtained using such methods may be degraded due to long incubation times or exposure to harsh conditions.

Physical disruption methods using tissue homogenizers, mortar and pestle, dounce homogenization, frozen pulverization, and bead beaters, have been employed with biological samples. The nucleic acid obtained using these methods, however, is typically degraded due to shearing, long incubation times and high temperatures that may be generated using these methods. Further, such methods present health and safety concerns as a portion of the sample may be aerosolized during the physical disruption procedure. This becomes a critical issue when the sample potentially contains infectious agents such as HIV, hepatitis, or other pathogenic microorganisms.

Thus, there is a need for methods and compositions for isolating high integrity, i.e., high molecular weight, nucleic acid molecules from biological samples in a safe, rapid, and efficient manner, especially from whole tissues.

Summary of the Invention

The present invention provides novel compositions, methods, and kits for releasing or for isolating nucleic acids from biological samples using at least one surfactant and at least one protease.

In one aspect, compositions are provided for releasing nucleic acids from a biological sample. In certain embodiments, these compositions include at least one cationic surfactant, at least one protease, and a buffer.

In another aspect, methods for releasing and methods for isolating nucleic acids from a biological sample are provided. In certain embodiments, these methods include combining the sample with at least one cationic surfactant, at least one protease, and a buffer to form a reaction composition. Optionally, the reaction composition further comprises a second surfactant and a salt. The composition is incubated under appropriate conditions to allow the sample to be digested, releasing the nucleic acid. In certain embodiments, the released nucleic acid is then isolated.

Kits for isolating and/or releasing nucleic acid from biological samples are also provided. In certain embodiments, the invention provides kits comprising at least one cationic surfactant and at least one protease. Kits further comprising a second surfactant, a salt, organic extraction agent(s), organic precipitating agent(s), solubilizing agents(s), or combinations of these components are also provided.

Brief Description of the Figures

Figure 1: represents the fluorescent intensity (measured in fluorescence units) of dye-labeled casein substrate incubated with Proteinase K at concentrations ranging from 40 fg/mL to 40 ug/mL.

Figure 2: illustrates the effect of various surfactants on Proteinase K digestion of a dye-labeled casein substrate. Figure 2A depicts a reaction composition comprising 20 g/ml Proteinase K; Fig. 2B depicts a reaction composition comprising 2.5 g/ml Proteinase K; Fig. 2C depicts a reaction composition comprising 1.25 g/ml Proteinase K. The tetraalkylammonium halide symbol (open diamond) represents the average data for the cationic surfactants dodecyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, cetyltrimethylammonium bromide, and mixed alkyltrimethylammonium bromide. The symbol for benzylalkylammonium halide (open circle) represents the average data for the cationic surfactants benzalkonium chloride, benzyldimethyldodecylammonium bromide, benzyldimethylhexadecylammonium bromide and benzyldimethyltetradecyl ammonium chloride. The symbol for Mackernium (X) represents the average data for the cationic surfactants Mackernium SDC-85, Mackernium KP, Mackernium WLE and Mackernium NLE. The polyquaternium symbol (closed circle) represents the average data for the cationic polymers Mackernium 006 and Mackernium 007. The results obtained using water (closed diamond) and using the anionic surfactant SDS (closed square) are also shown. The error bars represent the standard deviation for the each data set.

Figure 3: illustrates the quantity of nucleic acid, measured in g of nucleic acid/mg of sample, released using reaction compositions comprising Proteinase K and various surfactants.

Figure 4: depicts a stained agarose gel that provides a comparison between the amount of nucleic acid that is released (supe) with the nucleic acid remaining in the sample (pellet) following incubation with the reaction compositions comprising the indicated surfactants.

Figure 5: illustrates the quantity of nucleic acid that is released and isolated from liver slices incubated for 20 minutes at 45° C in reaction compositions comprising 2 mg/mL Proteinase K and the indicated surfactants. When used in these figures, NMP is 1-methyl-2-pyrolidinone.

Figure 6: depicts an ethidium bromide stained agarose gel that demonstrates the quality (integrity) of the released and isolated nucleic acid of Figure 5.

Figure 7: illustrates the quantity of nucleic acid that is released and isolated from liver slices incubated for 30 minutes at 45° C in reaction compositions comprising 1 mg/mL Proteinase K and the indicated surfactants and additives. When used in these figures, "nothing" means no additional reagent was added.

Figure 8: depicts an ethidium bromide stained agarose gel that demonstrates the quality (integrity) of the released and isolate nucleic acid of Figure 7.

Figure 9: illustrates the effect of exposing liver slices to pretreatment solutions prior to incubation for 30 minutes at 45° C in reaction compositions comprising 1 mg/mL

Proteinase K and the indicated surfactants and additives. When used in these figures, HTSP 2X means Nucleic Acid Purification Lysis Solution; AcrOr means acridine orange.

Figure 10: depicts a stained agarose gel that demonstrates the quality of nucleic acid released and isolated from samples that were incubated in pretreatment solutions prior to surfactant:Proteinase K treatment.

Figure 11: depicts a stained agarose gel that demonstrates the quality of nucleic acid released and isolated using reaction compositions comprising varying amounts of aurintricarboxylic acid.

Figure 12: depicts a stained agarose gel that provides a comparison of the amount and integrity of nucleic acid released and isolated with various cationic compounds.

Detailed Description of the Invention

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents cited in this application, including, but not limited to patents, patent applications, articles, books, and treatises, are expressly incorporated by reference in their entirety for any purpose.

Definitions

The term "alkyl group" refers to a hydrocarbon moiety based on a linear, branched or cyclic alkane. Linear alkanes are organic compounds with the general chemical formula C_nH_{2n+2}, where C represents a carbon atom, H represents a hydrogen atom, and n represents a whole number. Exemplary linear alkanes include, but are not limited to, methane (CH_4), propane (C_3H_8), butane (C_4H_{10}), octane and the like. Exemplary branched alkanes include, but are not limited to, isobutane (C_4H_{10}),

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isopentane (C_5H_{12}), and the like. Exemplary cyclic alkanes include, but are not limited to, cyclobutane (C_4H_8), cyclohexane (C_6H_{12}), and the like. Exemplary alkyl groups include, but are not limited to, methyl ($-CH_3$), propyl ($-C_3H_7$), octyl ($-C_8H_{17}$), and the like.

5   While alkanes are typically unreactive, alkyl groups can be combined with other molecules to form compounds. For example, isopropyl alcohol (C_3H_7OH) is an alcohol that contains a propyl group. A general discussion of alkyl groups and alkanes can be found, among other places, in Morrison and Boyd, Organic Chemistry, 3rd Ed., Allyn and Bacon, Boston, MA, 1973; and Vollhardt, Organic Chemistry, W.H. Freeman, New York, NY, 1987.

10   The term "aryl group" refers to a hydrocarbon moiety that is based on benzene or other aromatic compounds. Aryl groups, like alkyl groups, can be combined with other molecules to form compounds. A general discussion of aromatic compounds and aryl groups can be found, among other places, in Morrison and Boyd, Organic Chemistry, 3rd Ed., Allyn and Bacon, Boston, MA, 1973; and Vollhardt, Organic Chemistry, W.H.

15   Freeman, New York, NY, 1987, particularly Chapter 19.

The term "biological sample" is used in a broad sense and is intended to include a variety of biological sources that contain nucleic acids. Such sources include, without limitation, whole tissues, including biopsy materials and aspirates; in vitro cultured cells, including primary and secondary cells, transformed cell lines, and tissue and cellular

20   explants; whole blood, red blood cells, white blood cells, and lymph; body fluids such as urine, sputum, semen, secretions, eye washes and aspirates, lung washes and aspirates; and the like. Microorganisms and viruses that may be present on or in a biological sample are within the scope of the invention.

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The term "buffer," as used herein, refers to aqueous solutions or compositions that resist changes in pH when acids or bases are added to the solution. This resistance to pH change is due to the solution's buffering action. Solutions exhibiting buffering activity are referred to as buffers or buffer solutions. Buffers typically do not have an unlimited ability to maintain the pH of a solution or composition. Rather, typically they are able to maintain the pH within certain ranges, for example between pH 5 and pH 7. *See, generally,* C. Mohan, Buffers, A guide for the preparation and use of buffers in biological systems, Calbiochem, 1999. Exemplary buffers include, but are not limited to, MES ([2-(N-Morphilino)ethanesulfonic acid]), ADA (N-2-Acetamido-2-iminodiacetic acid), and Tris ([*tris*(Hydroxymethyl)aminomethane]; also known as Trizma); Bis-Tris; ACES; PIPES; MOPS; and the like (all available from Sigma).

Buffers that maintain the pH within a certain pH range, for example, between pH 5 and pH 7, and similar terms as used herein, are intended to encompass any buffer that exhibits buffering action at some point within the stated pH range. Thus, that term encompasses buffers that do not exhibit buffering capacity within the entire stated range, and buffers with buffering capacity that extend beyond the stated range. For example, solution A may exhibit buffering capacity between pH 5.2 and 6.7, solution B may exhibit buffering capacity between 6.0 and 8.0. For purposes of this invention, both of those solutions would be considered buffers that maintain the pH within the range of pH 5.0 to pH 7.0. The skilled artisan will be able to identify an appropriate buffer for maintaining the pH between a specified range using a buffer table. Buffer tables can be found in, among other places, the Calbiochem 2000-2001 General Catalog at pages 81-82, and the Sigma 2000-2001 Biochemicals and Reagents for Life

Science Research Catalog at page 1873, both of which are expressly incorporated by reference.

The term "chaotrope" or "chaotropic salt," as used herein, refers to a substance that causes disorder in a protein or nucleic acid by, for example, but not limited to, altering the secondary, tertiary, or quaternary structure of a protein or a nucleic acid while leaving the primary structure intact. Exemplary chaotropes include, but are not limited to, guanidine hydrochloride, guanidinium thiocyanate, sodium thiocyanate, sodium iodide, sodium perchlorate, urea, and the like. A typical anionic chaotropic series, shown in order of decreasing chaotropic strength, includes: $CCl_3COO^- >> CNS^- > CF_3COO^- > ClO_4^- > I^- > CH_3COO^- > Br^-$, Cl^-, or CHO_2^-. Descriptions of chaotropes and chaotropic salts can be found, among other places, in Hamaguchi, Kozo and Geiduschek; E. Peter; Hatefi, Y. and Hanstein, W.G. (1962), "Solubilization of Particulate Proteins And Nonelectrolytes by Chaotropic Agents," Proc. Natl. Acad. Sci. 62: 1129-1136); The Effect Of Electrolytes On The Stability Of The Deoxyribonucleate Helix, J. Amer. Chem. Soc. 84: 1329-1338); and U.S. Patent No. 5,234,809.

A "chelator" is a cyclic or heterocyclic ring compound that is capable of binding to metal cations, such as sodium (Na^+), magnesium (Mg^{++}), or calcium (Ca^{++}) ions. At least two non-metal ions of the chelator form coordinate bonds with the metal cation to incorporate it into the ring. Thus, the metal cation is no longer free to participate in other reactions. Cations that have a single charge, such as Na^+ are referred to as monovalent cations, while those with a double charge, such as Mg^{++} or Ca^{++}, are referred to as divalent cations. Some enzymes, such as certain polymerases and nucleases typically function in the presence of free divalent cations. Thus, a divalent

cation chelator, provided in sufficient amounts, typically would inhibit or reduce the activity of such divalent cation-dependent enzymes. Exemplary chelators include, but are not limited to, EDTA, EGTA, diaminoethane, and the like.

A "nuclease," such as a deoxyribonuclease (DNAse) or a ribonuclease (RNAse), is an enzyme that catalyzes the hydrolysis of phosphodiester linkages in nucleic acid polymers. Nucleases cause nucleic acid polymers to degrade, releasing constituent nucleotides and/or oligonucleotides (fragments of the polymers). A DNAse degrades DNA molecules and an RNAse degrades RNA molecules. Nuclease activity can typically be slowed or prevented by appropriate nuclease inhibitors.

As used herein, the term "organic solvent" refers to organic liquids, i.e., those comprising molecules with a hydrocarbon backbone. Organic solvents are capable of solvating non-solvent molecules, e.g., by surrounding them with solvent molecules so that the non-solvent molecules are dissolved in the solvent. Exemplary organic solvents include, but are not limited to, benzene, carbon tetrachloride, chloroform, phenol and other alcohols such as ethanol, methanol, and isopropanol (2-propanol), dimethyl sulfoxide (DMSO), and the like. Organic solvents can be used, for example, to extract nucleic acids from certain biochemical compositions, or to precipitate nucleic acids from aqueous solutions. Discussions of organic solvents can be found, among other places, in Morrison & Boyd, Organic Chemistry, 3d Edition, Allyn & Bacon, Boston, MA, 1973.

As used herein, the term "protease" refers to an enzyme that catalyzes the cleavage of peptide bonds, e.g., in proteins, polypeptides, oligopeptides, and peptides (collectively "peptides"). Exemplary proteases include, but are not limited to, subtilisins,

subtilases, alkaline serine proteases, and the like. Subtilases are a family of serine

proteases, i.e., enzymes that cleave peptides after the amino acid serine. Subtilases

are found in prokaryotic and eukaryotic organisms, such as bacteria, fungi, yeast, and

higher organisms. Subtilisins are bacterial serine proteases that have broad substrate

5   specificities. Subtilisins are relatively resistant to denaturation by chaotropes, such as

urea and guanidine hydrochloride, and anionic detergents, such as sodium dodecyl

sulfate. Exemplary subtilisins include, but are not limited to: Proteinase K; Proteinase

R; Proteinase T (isolated from *Tritirachium album* Limber); Subtilisin DY, Carlsberg,

also referred to as Subtilisin, Subtilisin A, Subtilopeptidase A, or Alcalase Novo; BPN',

10 also referred to as Nagarse proteinase, Nagarse, or Subtilopeptidase C; Novo, also

referred to as Bacterial proteinase Novo, Subtilisin B, or Subtilopeptidase B;

mesentericopeptidase; Thermitase; and the like. Discussions of subtilases, subtilisins,

Proteinase K, and other proteases may be found, among other places, in Genov et al.,

(1995) Stability Of Subtilisins And Related Proteinases (Subtilases), Int. J. Peptide

15 Protein Res. 45: 391-400); Narhi and Arakawa (1989) Sodium Dodecyl Sulfate

Polyacrylamide Gel Electrophoresis As A Method For Studying The Stability Of

Subtilisin, Biochimica et Biophysica Acta. Vol. 990: 144-149); Dixon and Webb,

Enzymes, 3d Edition, Academic Press, New York, NY (1979); and Creighton, Proteins:

Structures and Molecular Principles, W.H. Freeman and Co., New York, NY (1984).

20   The term "salt" as used herein, refers to a compound produced by the interaction

of an acid and a base. Exemplary salts include, but are not limited to, sodium chloride

(table salt), potassium phosphate, sodium bicarbonate, and the like. In water and other

aqueous solutions, salts typically dissociate into an "anion" or negatively charged

subcomponent, and a "cation" or positively charge subcomponent. For example, when sodium chloride (NaCl) is dissolved in water, it dissociates into a sodium cation (Na^+) and a chloride anion (Cl^-).

As used herein, the term "surfactant" refers to a surface-active agent that generally comprises a hydrophobic portion and a hydrophilic portion. Examples of surfactants include, but are not limited to, detergents and bile acid salts. (*See*, e.g., Bhairi, A Guide to the Properties and Uses of Detergents in Biological Systems, Calbiochem-Novabiochem Corp. 1997). Surfactants may be categorized as anionic, nonionic, zwitterionic, or cationic, depending on whether they comprise one or more charged group. Anionic surfactants, such as SDS or lauryl sarkosine, contain a negatively charged group and have a net negative charge. Nonionic surfactants contain non-charged polar groups and have no charge. Exemplary nonionic surfactants include, but are not limited to, Tween 20, Triton X-100, NP-40, and the like. A zwitterionic surfactant contains both a positively charged group and a negatively charged group, and thus they are also uncharged.

A "cationic surfactant" has a positively charged group under the conditions examined. Cationic surfactants may contain quaternary amines or tertiary amines. Exemplary quaternary amine surfactants include, but are not limited to, cetylpyridinium chloride, cetyl trimethyl ammonium bromide (CTAB; Calbiochem #B22633 or Aldrich #85582-0), cetyl trimethyl ammonium chloride (CTACl; Aldrich #29273-7), dodecyl trimethyl ammonium bromide (Sigma #D-8638), octyl trimethyl ammonium bromide, tetradecyl trimethyl ammonium bromide, octadecylyl trimethyl ammonium bromide, stearoalkonium chloride, olealkonium chloride, cetrimonium chloride, alkyl trimethyl

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ammonium methosulfate, palmitamidopropyl trimethyl chloride, quaternium 84

(Mackernium NLE; McIntyre Group, Ltd.), wheat lipid epoxide (Mackernium WLE;

McIntyre Group, Ltd.), and the like. Exemplary ternary amine surfactants include, but

are not limited to, octyldimethylamine, decyldimethylamine, dodecyldimethylamine,

tetradecyldimethylamine, hexadecyldimethylamine, octyldecyldimethylamine,

octyldecylmethylamine, didecylmethylamine, dodecylmethylamine, triacetylammonium

chloride, cetrimonium chloride, alkyl dimethyl benzyl ammonium chloride, and the like.

The term "whole tissue" according to the present invention is used in the broad

sense to include any collection of cells organized to perform a specific function.

Examples of tissues include, without limitation: muscles, including cardiac, striated, and

smooth muscle; organs, such as kidney, liver, spleen, brain, etc.; nerves; dermal and

epidermal layers, such as skin; connective tissue such as bone, cartilage, ligaments,

and tendons; and the like. Fragments, pieces, sections, slices, and sub-components of

whole tissues are within the scope of the invention. The term whole tissue is not

limited, for example, to an entire organ or bone. Any aggregate or assembly of cells at

any level is, for purposes of this invention, considered whole tissue. Plant tissues, such

as leaves, roots, stems, and the like, are also within the scope of the present invention.

The term "nucleic acid," as used herein, refers to a polymer of ribonucleosides or

deoxyribonucleosides comprising phosphodiester linkages between subunits. Such

nucleic acids include, but are not limited to, genomic DNA, hnRNA, mRNA, rRNA,

tRNA, fragmented nucleic acid, nucleic acid obtained from subcellular organelles such

as mitochondria or chloroplasts, and nucleic acid obtained from microorganisms or DNA

or RNA viruses that may be present on or in a biological sample.

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Solid phase components that are capable of binding to nucleic acids released from a biological sample include a variety of materials that are capable of binding nucleic acids under suitable conditions. Exemplary solid phase components include, but are not limited to, silica particles, silicon dioxide, diatomaceous earth, glass, alkylsilica, aluminum silicate, borosilicate, nitrocellulose, diazotized paper, hydroxyapatite, nylon, metal oxides, zirconia, alumina, diethylaminoethyl- and triethylaminoethyl-derivatized supports (Chromegabond SAX, LiChrosorb-AN, Nucleosil SB, Partisil SAX, RSL Anion, Vydac TP Anion, Zorbax SAX, Nucleosil NMe_2, Aminex A-series, Chromex, and Hamilton HA Ionex SB, DEAE sepharose, QAE sepharose), hydrophobic chromatography resins (such phenyl- or octyl-sepharose), and the like.

Certain polymers, under appropriate conditions, are capable of precipitating nucleic acids from solution. Some polymers are capable of forming a nucleic acid-polymer complex that has a low solubility or is insoluble, so that complexed nucleic acid may precipitate. Examples of polymers that form such complexes with nucleic acids include, but are not limited to, polyethyleneimine, DEAE dextran, polylysine, polyarginine, polyhistidine, other weakly basic polymers (described in, among other places, U.S. Patent Nos. 5,582,988; 5,733,762; 5,622,822; and 5,599,667), and the like. Other polymers, such as polyethylene glycol, bind significant amounts of water in a solution, which may also cause nucleic acids to precipitate from the solution.

Exemplary Embodiments of the Invention

The present invention is directed to compositions, methods, and kits for isolating nucleic acid from biological samples, preferably whole tissue. The compositions, methods, and kits of the invention reduce the time needed for sample preparation,

decrease potential safety risks posed by multi-step procedures that require repeated sample manipulation, and provide high integrity (i.e., minimally degraded) high molecular weight nucleic acid.

The compositions and kits comprise and the methods employ surfactants and proteases. The biological sample is combined with at least one cationic surfactant and at least one protease to form a reaction composition. The reaction composition is then incubated in a manner such that nucleic acids are released from the biological samples. For example, during incubation, these reaction compositions typically can: (i) macerate or disaggregate the biological sample; (ii) lyse the cells comprising the sample; (iii) sterilize the sample; (iv) neutralize or inactivate nucleases; and (v) release nucleic acids from the biological sample.

While Proteinase K alone will effectively macerate whole tissue when incubated for approximately 12-18 hours, the released nucleic acid is frequently highly degraded. This degradation is due, at least in part, to nucleases present within the sample. The maceration process can be accelerated, as disclosed herein, by the addition of a cationic surfactant to the reaction composition. Under appropriate conditions, in certain embodiments, high integrity nucleic acid can be efficiently obtained from biological samples in 60 minutes or less using the compositions and methods of the invention. Thus, the compositions and methods of the invention provide an unexpected advantage.

Some cationic surfactants, under appropriate conditions, form a complex with the released nucleic acid and precipitate. These cationic surfactant:nucleic acid complexes

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may be dissolved using a nonionic surfactant and an appropriate salt. Thus, in certain embodiments, the compositions further comprise a second surfactant and a salt.

In certain embodiments, the released nucleic acid is isolated. Nucleic acids can be isolated effectively from aqueous solutions that contain biomolecules, such as lipids, proteins, and the like, using organic solvents. For example, organic solvents such as phenol or combinations of phenol and chloroform can be used to extract the nucleic acids from such solutions. The extracted nucleic acids can be isolated by precipitating the nucleic acid using, for example, alcohols such as ethanol, propanol, or butanol. In other embodiments, the released nucleic acid is not isolated from the reaction composition.

Nucleic acids may also be isolated from the reaction composition using certain polymers or divalent cations for precipitating the nucleic acids. Exemplary polymers for precipitating nucleic acids include, but are not limited to, polyethylene glycol, polyethyleneimine, DEAE dextran, polylysine, polyarginine, polyhistidine, and other weakly basic polymers. Descriptions of such polymers may be found, among other places, in U.S. Patent Nos. 5,582,988; 5,733,762; 5,622,822; and 5,599,667. Certain divalent cations may also cause nucleic acid molecules to selectively precipitate from solution. Examples of such divalent cations include, but are not limited to, zinc chloride, magnesium chloride and magnesium sulfate.

Additionally, nucleic acids may be isolated using solid phase supports that selectively bind nucleic acids. For example, but not limited to, nucleic acids from a sample can be adsorbed to a solid phase in the presence of high concentrations of a chaotrope or salt. The solid phase may be washed to remove contaminating material,

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and the nucleic acid can be eluted from the solid phase using a solution with a low ionic strength. Suitable solid phases include, but are not limited to, silica particles, silicon dioxide, diatomaceous earth, glass, alkylsilica, aluminum silicate, or borosilicate.

Also, nucleic acids can be bound to an ion exchange resin in the presence of low

5   ionic strength. Following removal of the contaminating components by washing, the nucleic acid is eluted from the solid phase by increasing the ionic strength. Exemplary ion-exchange resins include, but are not limited to, diethylaminoethyl- and triethylaminoethyl-derivatized supports (Chromegabond SAX, LiChrosorb-AN, Nucleosil SB, Partisil SAX, RSL Anion, Vydac TP Anion, Zorbax SAX, Nucleosil NMe_2, Aminex A-

10   series, Chromex, and Hamilton HA Ionex SB, DEAE sepharose, or QAE sepharose, and the like). The skilled artisan will appreciate that other solid phase materials may also be used, for example, but not limited to, nitrocellulose, diazotized paper, hydroxyapatite, nylon, metal oxides, zirconia, alumina, and reverse-phase resins (such octyl or phenyl sepharose).

15   The compositions of the invention include at least one protease. In certain embodiments, proteases such as subtilisins, subtilases, and alkaline serine proteases are employed. Exemplary proteases include, but are not limited to, Proteinase R, Proteinase T, subtilisin DY, dispase, subtilisin Carlsberg, subtilopeptidase A, thermolysin, thermostable proteases (such as those from *Thermus* Rt41A and *Bacillus*

20   *thermoproteolyticus rokko*), and alkaline serine proteases form *Streptomyces griseus* or *Bacillus licheniformis*. A preferred protease, according to certain embodiments, is Proteinase K.

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Certain proteases, including Proteinase K, are typically stabilized by the presence of calcium chloride. When calcium chloride is combined with certain anionic surfactants, such as SDS, a relatively insoluble precipitate can be formed. To avoid such precipitation, scientists sometimes omit calcium chloride when employing both protease and anionic surfactants. In preferred embodiments of the invention in which no substantial amount of anionic surfactants are employed, calcium chloride may be included.

To obtain high integrity DNA, in certain embodiments, DNAse inhibitors may be added to the compositions of the invention. For example, many endogenous DNAses are inhibited by divalent cation chelators, such as EDTA (ethylenediaminetetraacetic acid), EGTA ([ethyleneglycol-bis(-aminoethyl)-N,N,N',N'-tetraacetic acid]), DTPA (diethylenetriaminepentaacetic acid), and the like.

In certain embodiments, the at least one cationic surfactant comprises a quaternary amine, a tertiary amine, or both. In certain embodiments, the at least one cationic surfactant comprises cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTACl), hexadecyltrimethylammonium bromide (HDTAB), or hexadecyltrimethylammonium chloride (HDTACl), and the at least one protease comprises Proteinase K.

In certain embodiments, isolating the nucleic acid comprises extracting the nucleic acid with an organic agent, such as, but without limitation, phenol, chloroform, or both phenol and chloroform. In certain embodiments, the nucleic acid may be precipitated with an organic agent, for example, without limitation, an alcohol such as isopropanol, ethanol, or butanol. A combination of extracting the nucleic acid with an

organic agent and precipitating the extracted nucleic acid with an organic agent is also within the scope of isolating the nucleic acid.

In certain embodiments, methods for isolating ribonucleic acid from a biological sample are provided comprising using a cationic surfactant, a protease, and a buffer. In certain embodiments, the cationic surfactant is CTAB (Sigma or Aldrich), CTACl (Sigma or Aldrich), HDTAB (Sigma #H-5882 or Fluka #52367), or HDTACl (Fluka #41199). When high integrity RNA is desired, the presence of endogenous RNAses sometimes limit the efficiency of some current procedures. Two components of the instant compositions and methods, however, can counteract the activity of endogenous RNAses, enhancing the isolation of high integrity RNA. First, Proteinase K has been shown to inactivate RNAse. Second, cationic surfactants may form complexes with RNA, decreasing the susceptibility of the RNA to RNAse degradation. See, e.g., Dahl, C.E. and Macfarlane, D.E., Isolation Of RNA From Cells In Culture Using Catrimox-14™ Cationic Surfactant, BioTechniques Vol. 15, No. 6: 1102-1105 (1993); Macfarlane, D.E. and Dahle, C.E., Isolation RNA From Whole Blood -The Dawn Of RNA-Based Diagnosis? Nature, Vol. 362: 186-188 (1993); Macfarlane, D.E. and Dahle, C.E., Isolating RNA From Clinical Samples With Catrimox-14 and Lithium Chloride, Journal of Clinical Laboratory Analysis. 11: 132-139 (1997).

According to certain embodiments, endogenous RNAse activity is further diminished using the compositions and methods of the invention. For example, RNAses are less active at lower pH, e.g., between pH 5 and 7, and temperatures below 50° C. Acridine orange can also decreases the nucleolytic activity of RNAse, presumably by interacting with the released RNA molecules making them less susceptible to

degradation. RNAse inhibitors may also be added to limit or prevent RNAse activity. Exemplary RNAse inhibitors include, but are not limited to, aurintricarboxylic acid, vanadylate ribonucleoside complexes, phenylglyoxal, p-hydroxyphenylglyoxal, polyamines, spermidine, 9-aminoacridine, iodoacetate, bentonite, poly[2'-O-(2,4-dinitrophenyl)]poly(adenyhlic acid), zinc sulfate, bromopyruvic acid, formamide, dimethylformamide, copper, zinc, and the like. In certain embodiments, the compositions and methods of the invention comprise at least one RNAse inhibitor.

According to certain embodiments, cationic surfactants used in the compositions and methods have the general chemical formula:

$$R_3 - \overset{\overset{\displaystyle R_1}{|}}{\underset{\underset{\displaystyle R_4}{|}}{N+}} - R_2 \qquad X^-$$
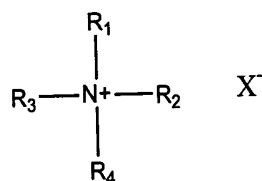
$(N^+R_1R_2R_3R_4 \text{:} X^-)$, where the cation moieties R_1, R_2, R_3, and R_4 independently may be: − H, an alkyl group containing up to 20 carbon atoms, or an aryl group containing between 6 and 26 carbon atoms; wherein at least one of R_1, R_2, R_3, or R_4 contains an alkyl group of at least 6 carbons; and where X^- is an anion. For example, the cationic surfactant may be an alkyltrimethyl ammonium salt, where R_1, R_2, and R_3 are methyl groups, and R_4 is an alkyl group comprising 6, 8, 10, 12, 14, 16, or 18 carbon atoms. The cationic subcomponent $(N^+R_1R_2R_3R_4)$ of the alkyltrimethyl ammonium salt might be (without limitation) a cetyltrimethylammonium group, a hexadecyltrimethylammonium group, a tetradecyltrimethylammonium group, a dodecyltrimethylammonium group, a lauryl trimethylammonium group, or the like. The anionic subcomponent (X^-) of this exemplary alkyltrimethyl ammonium salt might be (without limitation) any of the

following ions: bromide, chloride, iodide, hydroxide, nitrate, sulfate, phosphate, formate, acetate, propionate, oxalate, malonate, succinate, or citrate. In certain embodiments, the cationic surfactant is a benzyldimethyl-n-alkylammonium salt, comprising the same group of anions.

5   The compositions and methods of the invention may further comprise solubilizing agents. Solubilizing agents are particularly useful when the biological sample is resistant to digestion, for example, when the sample comprises whole tissue. In certain embodiments, solubilizing agents help to disassemble the intercellular matrix, allowing the surfactant and protease to more effectively penetrate and disaggregate the sample.

10   Exemplary solubilizing agents include, but are not limited to, 1-methyl-2-pyrolidinone, N-methyl pyrolidinone, pyrolidinone, dimethylsulfoxide, dimethylformamide, and the like.

The invention also provides kits designed for releasing and/or for isolating nucleic acid from biological samples. The kits comprise at least one cationic surfactant and at least one protease, and according to certain embodiments may include additional

15   component(s). In certain embodiments, the kits preferably contain components in pre-measured unit amounts to minimize the measurements by end-users. In certain embodiments, the kits preferably include instructions for performing one or more methods of the invention. Preferably, the kit components are optimized to operate in conjunction with one another.

20   In certain embodiments, the at least one surfactant comprises CTAB, CTACl, HDTAB, HDTACl, or an equivalent. In certain embodiments, the at least one protease comprises Proteinase K. Optionally, other reagents for isolating extracted nucleic acid may be included in such kits. The kits of the invention may further comprise reaction

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buffers, salts, ions, stabilizers, nuclease inhibitors, solubilizing agents, or combinations of these components.

The invention, according to certain embodiments, may be better understood by reference to examples. The following examples are intended for illustration purposes only, and should not be construed as limiting the scope of the invention in any way.

Example 1:

An assay was developed to measure the activity of Proteinase K with BoDipy-labeled Casein Conjugate (Enz Check Protease Assay Kit , Product number E6638; Molecular Probes, Eugene, OR). The concentration of fluorescent BoDipy moieties in this derivatized conjugate is sufficiently high so that when the protein is intact, the fluorescence of individual moieties is quenched. Upon digestion of the derivatized casein by a protease, however, peptides containing a lower number of BoDipy dye molecules are released, resulting in an increase in fluorescence. To determine an initial dynamic range for an assay, BoDipy-labeled Casein was digested for one hour with a decreasing concentration of Proteinase K as follows.

Proteinase K (Product number 2546, Ambion) was added over a range of final concentrations (40 fg/ml to 40 µg/mL) to 500 µL of buffer (10 mM Tris, pH 8, 20 mM $CaCl_2$) containing 10 µg/mL BoDipy-labeled Casein Conjugate. Following a one hour incubation at 60°C with mixing, 100 µL aliquots were transferred to a 96-well optical plate (MicroAmp® Optical 96-Well Reaction Plate, Applied Biosystems, Foster City, CA) and the fluorescence was measured in an ABI Prism 7700 spectrophotometer (Applied Biosystems). The background fluorescence was determined by incubating the substrate in buffer without Proteinase K.

As shown in Figure 1, the amount of fluorescence increased as the Proteinase K concentration increased. The signal appeared to plateau at around 30,000 fluorescence units and released fluorescence was detected from reaction compositions containing as little as 50 ng/mL Proteinase K.

Example 2:

Initially, a series of reaction compositions were analyzed for their ability to disaggregate a biological sample, e.g., slices of liver. These reaction compositions comprised 100 mM Tris, pH 8.0, 20 mM dithiothreitol (DTT), and optionally,1 mg/mL Proteinase K, surfactants (e.g., cationic, nonionic and anionic detergents), chaotropes, or the additive 1-methyl-2-pyrollidinone (Sigma). Each reaction compositions was placed in a tube with a slice of liver and then incubated at 65° C. The tubes were observed periodically to determine which reaction compositions were able to efficiently macerate the sample.

As shown in Table 1, Proteinase K alone was able to effectively digest the liver tissue, but only after extended incubation at 65° C. Of all of the reaction compositions examined, those containing the cationic surfactant CTAB most effectively macerated the sample during a one hour incubation period. Additionally, CTAB appeared to enhance the proteolyitic activity of Proteinase K.

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Table 1.

	65°C					
	No Proteinase K			1 mg/mL Proteinase K		
	2-5 hr	16-24 hr	72hr	1 hr	2-5 hr	16-24 hr
Cationic Surfactants						
Cetyltrimethylammonium bromide (1%)				100%		100%
Cetyltrimethylammonium bromide (0.1%)				100%		100%
Nonionic Surfactants						
Tween 80 (0.1%)				0%		0%
Tween 80 (1%)				0%		0%
Tween 80 (0.1%)				0%		
Triton X-100 (0.1%)				0%		0%
Triton X-100 (2%)	0%	0%				
Triton X-100 (1%)				30%		100%
Anionic Surfactants						
Chenodeoxycholic Acid (0.1%)				0%		0%
Chenodeoxycholic Acid (1%)				0%		0%
Chenodeoxycholic Acid (2%)		0%				
Cholic Acid (2%)		0%				
Glycocholic Acid (0.1%)				0%		0%
Glycocholic Acid (1%)				0%		0%
Taurochenodeoxycholic Acid (0.1%)				0%		0%
Taurochenodeoxycholic Acid (1%)				0%		0%
Taurocholic Acid (0.1%)				0%		0%
Taurocholic Acid (1%)				0%		0%
Taurocholic Acid (2%)		0%				
Taurodeoxycholic Acid (0.1%)				0%		0%
Taurodeoxycholic Acid (1%)				0%		0%
Octyl sulfonate (2%)			0%			
Hexane sulfonic acid (2%)			0%			
Octanonic Acid (2%)			0%			
SDS (1%)	0%				50%	
Lauryl Sarcosine (2%)			0%			
Saponin (0.1%)				0%		0%
Saponin (1%)				0%		0%
Saponin, (2%)	0%	50%				
NiaProof Type 8 (0.1%)				0%		0%
NiaProof Type 8 (1%)				0%		0%
Niaproof Type 8 (2%)	0%					
Chaotropes						
Guanidinium chloride (2M)	0%				0%	
Guanidinium thiocyanate (1M)	0%				25%	
Tetramethylammonium chloride (0.1M)		0%		0%		0%
Tetramethylammonium chloride (1M)		0%		0%		0%
Other additives						
1-methyl 2-pyrrolidinone (2%)		0%				
No Additives						
None					0%	75%

Example 3:

To test the effect of cationic surfactants on the activity of Proteinase K, a series of reaction compositions were prepared as follows. Reaction compositions comprised: Casein BoDipy conjugate (1 μg, 10 ng/μL), 10 mM Tris, pH 8, 20 mM $CaCl_2$, 1% surfactant (shown in Table 2 below) and a dilution series of Proteinase K at concentrations of 20 μg/mL, 5 μg/mL, 1.25 μg/mL, 0.31 μg/mL, 0.078 μg/mL, 0.02 μg/mL and 0.005 μg/mL. The anionic surfactant sodium dodecyl sulfate (SDS), known to activate Proteinase K activity under the conditions tested, was included as a positive control. Reaction tubes were incubated at 60°C in a 96-well optical plate (MicroAmp[®] Optical 96-Well Reaction Plate, Applied Biosystems) and the amount of released fluorescence was measured at the indicated time points using an ABI Prism 7700.

Table 2. Surfactants

Surfactant	Source
Olealkonium chloride	(CAS No.: 37139-99-4, McIntyre Group LTD).
Mackernium SDC-85 (Stearalkonium chloride)	(CAS No. 122-19-0, McIntyre Group LTD).
Mackernium NLE	(Quaternium-84)(McIntyre Group LTD)
Mackernium 006	(Polyquaternium-6) (Cas No. 26062-79-3) (McIntyre Group, LTD)
Mackernium 007	(Polyquaternium-7) (Cas No. 26590-05-6) (McIntyre Group, LTD)
Mackernium WLE	(Wheat Germamidopropyl Epoxy Q) (McIntyre Group, LTD)
Benzalkonium Chloride	(Sigma, Product Number B-6295)
Benzyldimethylhexadecylammonium Chloride	(Sigma, Product Number B-4136)
Benzyldimethyltetradecylammonium Chloride	(Sigma, Product Number B-5651)
Benzyldimethyldodecylammonium bromide	(Sigma, Product Number B-5776)
Tetradecyltrimethylammonium Bromide	(Sigma, Product Number T-4762)
Hexadecyltrimethylammonium Bromide	(Sigma, Product Number H-5882)
Dodecyltrimethylammonium Bromide	(Sigma, Product Number D-8638)
Mixed Alkyltrimethylammonium Bromide	(Sigma, Product Number M-7635)
Cetyltrimethylammonium bromide (CTAB)	(Calbiochem, Product number B22633)
Sodium Dodecyl Sulfate (SDS)	(Sigma, Product Number L6026)

As shown in Figures 2A-C: (i) Proteinase K without surfactant has high proteolytic activity, even at ambient temperature (see 0 minutes incubation in water); (ii)

SDS enhances the activity of Proteinase K on the casein substrate; (iii) the two cationic polymers (Mackernium 006 and 007) have a slightly inhibitory activity on Proteinase K activity; and (iv) all of the cationic surfactants tested appear to inhibit Proteinase K activity (compare the tetraalkylammonium halide, benzylalkylammonium halide, or polyquaterniums curve with that for water alone, i.e., no surfactant).

Example 4:

Since it is typically difficult to extract nucleic acid from whole tissue, it can be used effectively to illustrate the efficiency of the compositions and methods of the invention. Thus, the remainder of the examples were performed using liver tissue as an exemplary whole tissue. The skilled artisan will understand, however, that the disclosed compositions and methods may also be effectively employed using a broad range of biological samples and that the invention is not to be limited to use with any sample type.

In the initial approach for evaluating the efficacy of nucleic acid release by various test treatments, liver samples were digested for a specified period of time in reaction compositions comprising surfactant and Proteinase K. The protocol included removing undigested material using centrifugation. Preliminary results using purified nucleic acid and cationic surfactants demonstrated that the cationic surfactant was forming a precipitate with the nucleic acid (data not shown). Further, this complex was being removed with the undigested tissue during centrifugation. Thus, one way to quantify the amount of nucleic acid being released from the sample, included freeing the nucleic acid from the surfactant:nucleic acid complex. Conditions were evaluated for freeing the nucleic acid from the cationic surfactant complexes as follows.

Calf thymus DNA (Sigma; 700 µg/mL lightly sheared using an 18 gauge hypodermic needle) was mixed with 1% CTAB in 100 mM Tris, pH 8 to form a reaction composition. Initially, a noticeable precipitate was observed. A variety of additives, shown in Table 3, were tested with aliquots of this reaction composition to identify

5   compositions that would solubilize the cationic surfactant:nucleic acid precipitate.

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Table 3.

Sample	Additive (final concentration)	Result
1	91 mM NaCl	Nucleic acid precipitated
2	333 mM NaCl	Nucleic acid precipitated
3	117 mM Tetramethylammonium chloride	Nucleic acid precipitated
4	758 mM Tetramethylammonium chloride	Nucleic acid precipitated
5	95 mM Tetrabutylammonium chloride	Nucleic acid precipitated
6	400 mM Tetrabutylammonium chloride	Nucleic acid precipitated
7	312.5 mM NaCl, 6.25% 1-Methyl-2-Pyrrolidinone	Nucleic acid precipitated
8	692 mM Tetramethylammonium chloride, 7.5% 1-Methyl-2-pyrrolidinone	Nucleic acid precipitated
9	370 mM Tetrabutylammonium chloride, 7.4% 1-Methyl-2-pyrrolidinone	Nucleic acid precipitated
10	9.1% 1-Methyl-2-pyrrolidinone (Sigma #M-6762)	Nucleic acid precipitated
11	294 mM NaCl, 5.9% 1-Methyl-2-Pyrrolidinone, 5.9% Tween 20	Nucleic acid pellet fairly dissolved
12	643 mM Tetramethylammonium chloride, 7.0% 1-Methyl-2-pyrrolidinone, 7.0% Tween 20	Nucleic acid pellet fairly dissolved
13	345 mM Tetrabutylammonium chloride, 6.9% 1-Methyl-2-Pyrrolidinone, 6.9% Tween 20	Nucleic acid pellet began to solubilize
14	8.3% 1-Methyl-2-Pyrrolidinone, 8.3% Tween 20	Nucleic acid pellet began to solubilize
15	10% Tween 20 (Sigma #P-9416)	Nucleic acid precipitated
16	312.5 mM NaCl, 6.25% Tween 20	Nucleic acid pellet completely solubilized
17	2.0% Tween 20	Nucleic acid precipitated
18	160 mM NaCl, 1.6% Tween 20	Nucleic acid precipitated
19	9.1% 1-hexanesulfonic acid	Nucleic acid pellet partially solubilized
20	17% 1-hexanesulfonic acid	Nucleic acid pellet partially solubilized
21	200 mM NaCl, 16% Hexane sulfonic acid	Nucleic acid pellet partially solubilized
22	9.1% Decanesulfonic acid	Nucleic acid pellet partially solubilized
23	217 mM NaCl, 8.7% Decanesulfonic acid	Nucleic acid pellet partially solubilized
24	50% Nucleic Acid Purification Lysis Solution	Nucleic acid pellet completely solubilized
25	33% Nucleic Acid Purification Lysis Solution	Nucleic acid pellet completely solubilized
26	23% Nucleic Acid Purification Lysis Solution	Nucleic acid pellet partially solubilized
27	297 mM NaOAc	Nucleic Acid precipitated
28	292 mM NaOAc, 1.8% Tween 20	Nucleic Acid precipitated
29	313 mM NaOAc, 2.6% Tween 20	Nucleic Acid precipitated
30	535 mM NaCl, 1.75% Tween 20	Nucleic acid pellet completely solubilized

As shown in Table 3, four of the reaction compositions resulted in complete solubilization of the precipitate. Nucleic Acid Purification Lysis Solution (# 4305895, Applied Biosystems), used at final concentrations of 33% or 50%, completely solubilized

the precipitate (see samples 24-25). Additionally, some combinations of the salt NaCl and the nonionic detergent Tween 20 completely solubilized the precipitate (see samples 16 and 30). However, neither NaCl nor Tween 20, used alone, solubilized the precipitate (see samples 1, 2, 15, and 17). The skilled artisan will understand that

5   additional combinations of salts and nonionic surfactants used at varying concentrations can be used to dissolve the cationic surfactant:nucleic acid precipitates.

Example 5:

To evaluate the ability of various cationic surfactants to increase the digestion of tissue by Proteinase K, liver slices were digested using reaction compositions including

10   one of the sixteen surfactants (shown in Table 4), both with and without Proteinase K. The reducing agent dithiothreitol (DTT) was included to evaluate the effect of reducing conditions on tissue digestion. Liver tissue (80 - 120 mg) was placed into each of thirty-four Eppendorf tubes (seventeen pairs of tubes) containing 1000 μL of 10 mM Tris, pH 8, 20 mM $CaCl_2$, and 20 mM dithiothreitol. One of the sixteen surfactants was added to

15   one pair of tubes to yield a final concentration of 1% surfactant. The seventeenth pair of tubes served as non-surfactant controls. One milligram (mg) of Proteinase K was added to one tube of each of the seventeen pairs. The thirty-four tubes were then incubated at 60°C with mixing (Eppendorf Thermomixer, Model 5436).

Table 4.

Surfactant	Chemical Formula
SDS	$CH_3(CH_2)_{11}OSO_3Na$
Mackernium KP	$R_1R_2(CH_3)_2NCl$ R_1=fatty group, R_2=benzyl, methyl or epoxy group
Mackernium WLE	$R_1R_2(CH_3)_2NCl$ R_1=fatty group, R_2=benzyl, methyl or epoxy group
Benzalkonium chloride	$C_6H_5CH_2N(CH_3)_2RCl$ $R=C_8H_{17}$ to $C_{18}H_{37}$
Mackernium 006	Polyquaternium
Mackernium 007	Polyquaternium
Mackernium NLE	$R_1R_2(CH_3)_2NCl$ R_1=fatty group, R_2=benzyl, methyl or epoxy group
Mackernium SDC-85	$R_1R_2(CH_3)_2NCl$ R_1=fatty group, R_2=benzyl, methyl or epoxy group
Dodecyltrimethylammonium bromide	$CH_3(CH_2)_{11}N(CH_3)_3Br$
Tetradecyltrimethylammonium bromide	$CH_3(CH_2)_{13}N(CH_3)_3Br$
Hexadecyltrimethylammonium bromide	$CH_3(CH_2)_{15}N(CH_3)_3Br$
CTAB	$CH_3(CH_2)_{15}N(CH_3)_3Br$
Mixed alkyltrimethylammonium bromide	$CH_3(CH_2)_nN(CH_3)_3Br$
Benzyldimethyldodecylammonium bromide	$C_6H_5CH_2N[(CH_2)_{11}CH_3](CH_3)_2Br$
Benzyldimethyltetradecyl ammonium chloride	$C_6H_5CH_2N[(CH_2)_{13}CH_3](CH_3)_2Cl$
Benzyldimethylhexadecylammonium bromide	$C_6H_5CH_2N[(CH_2)_{15}CH_3](CH_3)_2Br$

Each tube was visually evaluated after 15 minutes and 52 minutes of incubation.

The results, shown in Table 5 (fifteen minutes incubation) and Table 6 (fifty-two minutes

incubation), indicate that under the conditions tested, the combination of CTAB and

Proteinase K caused total disaggregation of the tissue sample at either time point.

Notably, CTAB alone caused no apparent sample disaggregation. The presence of

DTT did not seem to significantly effect tissue disaggregation under these conditions.

Table 5. Observations after Fifteen Minutes Incubation.

Surfactant	PK	Tissue Disaggregation	Color of Composition
Buffer alone (control)	Y	0	Light pink
SDS	Y	3	Slightly Cloudy
Mackernium KP	Y	1	Slightly Cloudy
Mackernium WLE	Y	0	Clear
Benzalkonium chloride	Y	1	Slightly Cloudy
Mackernium 006	Y	1	Slightly Cloudy
Mackernium 007	Y	2	Slightly Cloudy
Mackernium NLE	Y	1	Slightly Cloudy
Dodecyltrimethylammonium bromide	Y	2	Medium Cloudy
Tetradecyltrimethylammonium bromide	Y	0	Clear
Hexadecyltrimethylammonium bromide	Y	0	Clear
CTAB	Y	4	Dark brown
Mackernium SDC-85	Y	3	Medium cloudy
Mixed alkyltrimethylammonium bromide	Y	3	Medium cloudy
Benzyldimethyldodecylammonium bromide	Y	0	Clear
Benzyldimethylhexadecylammonium bromide	Y	0	Clear
Benzyldimethyltetradecyl ammonium chloride	Y	0	Clear
Buffer alone (control)	N	0	Pink
SDS	N	0	Slightly cloudy
Mackernium KP	N	0	Clear
Mackernium WLE	N	0	Pink
Benzalkonium chloride	N	0	Clear
Mackernium 006	N	0	Slightly pink
Mackernium 007	N	0	Slightly pink
Mackernium NLE	N	0	Slightly brown
Dodecyltrimethylammonium bromide	N	0	Slightly pink
Tetradecyltrimethylammonium bromide	N	0	Slightly pink
Hexadecyltrimethylammonium bromide	N	0	Clear
CTAB	N	0	Slightly pink
Mackernium SDC-85	N	0	Clear
Mixed alkyltrimethylammonium bromide	N	0	Clear
Benzyldimethyldodecylammonium bromide	N	0	Slightly cloudy
Benzyldimethylhexadecylammonium bromide	N	0	Clear
Benzyldimethyltetradecyl ammonium chloride	N	0	Clear

PK= presence or absence of Proteinase K in the reaction composition; Y= Proteinase K was present; N= Proteinase K was not present in the reaction composition. Tissue disaggregation: 0 = none; 1 = No fragmentation, supernatant slightly cloudy; 2 = Little fragmentation, supernatant slightly cloudy; 3 = More significant tissue supernatant, supernatant very cloudy; 4 = complete tissue aggregation with little insoluble material remaining.

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Example 6:

To examine the ability of cationic surfactants and Proteinase K to release high integrity nucleic acids from a biological sample, the released nucleic acid was isolated. Slices of liver tissue (90 – 200 mg per slice) were placed into each of twenty-two Eppendorf microfuge tubes. Four hundred μL of 50 mM Tris, pH 8, 20 mM $CaCl_2$, 1% surfactant, and 1 mg Proteinase K was added to each tube. The tubes were incubated at 60°C for 22 minutes with mixing (Eppendorf Thermomixer, Model 5436).

Following the incubation, Nucleic Acid Purification Lysis Solution (# 4305895, Applied Biosystems; final concentration 29%), sodium acetate (final concentration 214 mM) and glycogen (Mussel type VII, Sigma; final concentration143 $\mu g/mL$) was added to each tube to dissolve the surfactant:nucleic acid complexes. The tubes were centrifuged in an Eppendorf microfuge (Model 5415C) at 14,000 RPM for 5 minutes to pellet remaining tissue fragments. The resulting supernatant was extracted using 700 μL of phenol (Sigma) saturated with 10 mM Tris HCl, pH 8.0, and 1 mM EDTA (saturated phenol). The pelleted material was also extracted with saturated phenol to obtain nucleic acid if any remained in the undigested sample. The extracted nucleic acid was precipitated using an equal volume (750 μL) of 2-propanol, and the tubes were incubated at –20°C for 3 hours. The samples were centrifuged at ambient temperature in a microfuge at 14,000 RPM for 5 minutes, as before, to pellet the nucleic acid. The nucleic acid pellets were washed with 1 mL of 70% ethanol and the samples were centrifuged at ambient temperature in a microfuge at 14,000 RPM for 5 minutes. The washed nucleic acid pellets were resuspended in 100 μL 10 mM Tris, pH 8, 0.1 mM EDTA, and 1 unit/μL RNasin. The amount of released nucleic acid was quantitated

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using a UV/Vis Spectrophotometer (Hewlett Packard, Model 8453). For quantitation purposes, it was assumed that the material that absorbed UV light at a wavelength of 260 nm was nucleic acid. Thus, an extinction coefficient of 1 OD_{260} = 40 µg/mL was used.

Figure 3 shows the amount of A_{260}-absorbing material recovered from the sample (µg nucleic acid/mg tissue processed). The results were normalized for the weight of each sample. Despite the reduced proteolytic activity of Proteinase K in the presence of the cationic surfactants, as demonstrated in the previous example, the amount of A_{260}-absorbing material was similar to the amount obtained with the anionic surfactants or without surfactant.

To evaluate the integrity of the released nucleic acid (i.e., its degree of degradation), and the amount of nucleic acid remaining in the tissue pellet, one tenth of each sample was analyzed by electrophoresis on a 1% agarose gel according to standard molecular biology procedures. Descriptions of standard molecular biology procedures may be found, among other places, in Molecular Cloning: A Laboratory Manual, Sambrook et al., 1989, Cold Spring Harbor Press, Cold Spring Harbor, NY; Current Protocols in Molecular Biology, Ausbel et al., 1993 and supplements through September 2000, John Wiley & Sons, New York, NY; or Molecular Biology Techniques, W. Ream et al., Academic Press, San Diego, CA, 1999.

As shown in Figure 4, most of the cationic surfactant:Proteinase K combinations released nucleic acid as evidenced by the little nucleic acid that remained in the pellet. The released nucleic acid was observed to have a high electrophoretic mobility, since it appeared near the bottom of the gel. Since intact, high integrity nucleic acid typically

has a low, rather than a high, electrophoretic mobility, the recovered nucleic acid apparently was highly fragmented using these conditions. In other words, it apparently had a low integrity.

Example 7:

The results from Example 6 indicate that there was significant nuclease activity present in the sample. To reduce the nucleolytic activity of endogenous RNAses, the pH of the reaction composition was lowered to approximately 6.0 and the incubation temperature was decreased to 45° C. Several additives, including phenylglyoxal (Aldrich #14243-3), 1-methyl-2-pyrolidinone, RNA Later (Ambion, #7020), and Streck Tissue Fixitive (Streck Laboratories, # 265138) were also tested to evaluate their effect on endogenous RNAse activity in these reaction compositions. Phenylglyoxal is known to reduce ribonuclease activity (*see* Takahashi, The Structure And Function Of Ribonuclease T_1. XI. Modification Of The Single Arginine Residue In Ribonuclease T1 By Phenylglyoxal And Glyoxal, J. Biochem (Japan) 68:659-664, 1970). The product literature for RNA Later claims that the reagent stabilizes RNA in tissue and Streck Tissue Fixative is believed to preserve the integrity of tissue.

Slices of liver tissue (100 - 200 mg/sample) were placed into nineteen Eppendorf tubes. Four hundred microliters of a reaction composition comprising 100 mM MES (Sigma #M-5287), pH 6, 20 mM $CaCl_2$, and 1 mg Proteinase K, was added to each tube. Additional components, including anionic surfactants (SDS, Sarkosyl) and cationic surfactants (CTAB, CTACl, and tetramethyl ammonium chloride) were added to specific tubes, as shown in Table 7.

Table 7.

Tube #	Additional Reaction Composition Components
1	No additional components (control)
2	1% Sodium Dodecyl Sulfate (SDS)
3	1% SDS, 10% 1-Methyl-2-pyrolidinone
4	1% SDS, 100 mM Phenylglyoxal
5	1% SDS, 80% RNA Later
6	1% SDS, 80% Streck Tissue Fixative
7	1% Cetyltrimethylammonium bromide (CTAB)
8	1% CTAB, 10% 1-Methyl-2-pyrolidinone
9	1% CTAB, 100 mM Phenylglyoxal
10	1% CTAB, 80% RNA Later
11	1% CTAB, 80% Streck Tissue Fixative
12	1% Sarkosyl
13	1% Sarkosyl, 10% 1-Methyl-2-pyrolidinone
14	1% Sarkosyl, 10 mM Phenylglyoxal
15	1% Sarkosyl, 80% RNA Later
16	1% Sarkosyl, 80% Streck Tissue Fixative
17	1% Cetyltrimethylammonium chloride (CTACl)
18	600 mM Tetrabutylammonium chloride
19	800 mM Tetramethylammonium chloride

The tubes were incubated at 45°C for 20 minutes with mixing (Eppendorf Thermomixer, Model 5436). Following the incubation, 171 μL of a solution containing 1.75 M NaCl, 29% Tween 20, 585 μg/mL glycogen (447 mM NaCl, 7.5% Tween 20, and 149 μg/mL glycogen) was added to each tube to dissolve the surfactant:nucleic acid complex. Glycogen served as a nucleic acid carrier. Undigested tissue fragments were removed by centrifugation using the methods the same as or similar to those discussed in Example 6. The supernatant was phenol extracted (saturated with 10 mM Tris HCl, pH 8, 1 mM EDTA; Sigma # P-4557), isopropanol precipitated (Aldrich #19076-4), and ethanol washed using methods the same as or similar to those described in Example 6. The washed nucleic acid pellets were resuspend with 100 μL 10 mM Tris, pH 8.0, 0.1 mM EDTA, and 1 unit/μL Rnasin. The amount of nucleic acid released and its integrity were evaluated as described in Example 6.

As shown in Figure 5, under these conditions, Proteinase K combined with either of the cationic surfactants (CTAB and CTACl) or the anionic surfactants (SDS and Sarkosyl) released A_{260} absorbing material from the sample. When either Streck Tissue Fixative or RNA Later was added to these reactions, very little A_{260} absorbing material was released. The average molecular weight for the released nucleic acid was typically higher when Proteinase K digestion was carried out at the lower pH and temperatures (compare Figure 4 to Figure 6). Additionally, the average molecular weight for the released nucleic acid was typically higher when Proteinase K digestion was performed in the presence of the cationic surfactants (compare Lanes 5-9 and 15-19 to 10-14 in Figure 6).

Example 8:

To compare CTACl to CTAB, those two surfactants were evaluated in parallel along with hexadecyltrimethylammonium bromide (HDTAB), hexadecyltrimethylammonium chloride (HDTACl), and Sarkosyl. Three additives, phenylglyoxal, acridine orange (e.g., Sigma #A6014), and 1-methyl-2-pyrolidinone, were also evaluated. Phenylglyoxal was tested to determine if it would reduce RNAse activity. Acridine Orange, a dye compound known to interact with nucleic acid molecules, was tested to determine if it would reduce nuclease activity. 1-Methyl-2-pyrrolidinone was tested to see if it would enhance solubility of the sample by various reaction compositions.

Slices of liver tissue (100 - 200 mg/sample) were placed into twenty-one Eppendorf tubes. Four hundred microliters of a reaction composition, comprising 100

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mM MES, pH 6, 20 mM $CaCl_2$, and 1 mg Proteinase K, was added to each tube.

Additional components were added to specific tubes, as shown in Table 8.

Table 8.

Tube #	Additional Reaction Composition Components
1	1% Cetyltrimethylammonium Bromide (CTAB)
2	1% CTAB, 100 mM Phenylglyoxal
3	1% CTAB, 10 % 1-Methyl-2-pyrolidinone
4	1% CTAB, 200 µg/mL Acridine orange
5	1% Cetyltrimethylammonium Chloride (CTACl)
6	1% CTACl, 100 mM Phenylglyoxal
7	1% CTACl, 10% 1-Methyl-2-pyrolidinone
8	1% CTACl, 200 µg/mL Acridine orange
9	1% Hexatrimethylammonium Bromide (HTMAB)
10	1% HTMAB, 100 mM Phenylglyoxal
11	1% HTMAB, 10% 1-Methyl-2-pyrolidinone
12	1% HTMAB, 200 µg/mL Acridine orange
13	1% Hexatrimethylammonium Chloride (HTMACl)
14	1% HTMACl, 100 mM Phenylglyoxal
15	1% HTMACl, 10% 1-Methyl-2-pyrolidinone
16	1% HTMACl, 200 µg/mL Acridine orange
17	1% Sarkosyl (N-lauryl sarcosine)
18	1% Sarkosyl, 100 mM Phenylglyoxal
19	1% Sarkosyl, 10% 1-Methyl-2-pyrolidinone
20	1% Sarkosyl, 200 µg/mL Acridine orange
21	No additives

5   The tubes were incubated at 45°C for 30 minutes with mixing (Eppendorf

Thermomixer, Model 5436). Surfactant:protease complexes were solubilized using

methods the same as or similar to those described in Example 7. Remaining tissue

fragments were removed by centrifugation using methods the same as or similar to

those described in Example 7. The supernatant was phenol extracted, isopropanol

10   precipitated, and ethanol washed using methods the same as or similar to those

described in Example 6. The washed nucleic acid pellets were resuspended and the

amount and the integrity of the released nucleic acid were evaluated using methods the

same as or similar to those described in Example 6.

As shown in Figure 7, the effect of the three additives varied depending on the surfactant. For example, the amount of nucleic acid released using CTACl and 1-methyl-2-pyrolidinone was greater in comparison with a composition of CTACl without an additive. However, the amount of nucleic acid released using HDTAB and 1-methyl-2-pyrolidinone was less in comparison with a composition of HDTAB without an additive.

When the released nucleic acid was analyzed by gel electrophoresis, high molecular weight nucleic acid was apparent in all reaction compositions containing HDTAB (see Figure 8). High molecular weight nucleic acid was also present in reaction compositions containing CTACl and either 1-methy-2-pyrolidinone or acridine orange. High molecular weight nucleic acid was also observed in the reaction composition comprising HDTACl and 1-methyl-2-pyrolidinone.

Example 9:

Example 7 demonstrated that the presence of certain reagents that have been used to stabilize RNA in tissue samples (*i.e.* Streck Tissue Fixative and RNA Later) inhibited the activity of Proteinase K. To attempt to reduce this inhibition, tissue was pre-incubated in several reagents to allow them to diffuse into the sample. Residual reagent was removed prior to incubation of the samples in reaction compositions.

Liver slices were incubated in various solutions prior to exposure to reaction compositions as follows. Slices of liver tissue (94 – 330 mg/sample) were placed into four sets of six Eppendorf tubes. Five hundred microliters of either Nucleic Acid Purification Lysis Solution, RNA Later, or Streck Tissue Fixative was added to one set

of tubes. The fourth set of tubes contained no pretreatment solution and served as a control.

The tubes were incubated for 4 hours at 4°C, then centrifuged in an Eppendorf microfuge (Model 5415C) at ambient temperature for 2 minutes at 14,000 RPM. The supernatant was discarded and four hundred microliters of a reaction composition comprising 100 mM MES, pH 6.0, 20 mM $CaCl_2$, and 1 mg Proteinase K, was added to each tube. One of the six additional components shown in Table 9 was also added to each tube. The tubes were incubated at 45°C with mixing (Eppendorf Thermomixer, Model 5436) for 30 minutes.

The resulting surfactant:protease complex was solubilized using methods the same as or similar to those described in Example 7. Remaining tissue fragments were removed by centrifugation using methods the same as or similar to those described in Example 7. The supernatant was phenol extracted, isopropanol precipitated, and ethanol washed using methods the same as or similar to those described in Example 6. The washed nucleic acid pellets were resuspended and the amount of nucleic acid released and the integrity of that nucleic acid were evaluated using methods the same as or similar to those described in Example 6.

Table 9.

Tube	Additional Reaction Composition Components
1	1% Cetyltrimethylammonium Bromide (CTAB)
2	1% CTAB, 10% 1-methyl-2-pyrolidinone (NMP)
3	1% CTAB, 200 µg/mL Acridine Orange
4	1% Cetyltrimethylammonium Chloride (CTACl)
5	1% CTACl, 10% NMP
6	1% CTACl, 200 µg/mL Acridine Orange

As shown in Figure 9, pretreatment of the samples with one of the pretreatment solutions resulted in a decreased amount of A_{260}-absorbing material that was released when compared to the control.

Example 10:

The ability of aurintricarboxylic acid to maintain the integrity of the nucleic acid released using reaction compositions comprising CTAB, CTACl, or SDS, was evaluated. Aurintricarboxylic acid is believed to inhibit the binding of proteins, e.g., nucleases, to nucleic acid and has been demonstrated to inhibit ribonucleases. Slices of liver tissue (77 - 186 mg/sample) were placed into twenty-one Eppendorf tubes. To each tube was added 400 µL of a reaction composition comprising 50 mM MES, pH 6.0, 20 mM $CaCl_2$, and additional components as shown in Table 10.

Table 10. Additional Reaction Composition Components

Tube	Additional Reaction Composition Components
1	1% CTAB, 5 mM Aurintricarboxylic Acid
2	1% CTAB, 2 mM Aurintricarboxylic Acid
3	1% CTAB, 1 mM Aurintricarboxylic Acid
4	1% CTAB, 0.5 mM Aurintricarboxylic Acid
5	1% CTAB, 0.2 mM Aurintricarboxylic Acid
6	1% CTAB, 0.1 mM Aurintricarboxylic Acid
7	1% CTAB
8	1% CTACl, 5 mM Aurintricarboxylic Acid
9	1% CTACl, 2 mM Aurintricarboxylic Acid
10	1% CTACl, 1 mM Aurintricarboxylic Acid
11	1% CTACl, 0.5 mM Aurintricarboxylic Acid
12	1% CTACl, 0.2 mM Aurintricarboxylic Acid
13	1% CTACl, 0.1 mM Aurintricarboxylic Acid
14	1% CTACl
15	1% SDS, 5 mM Aurintricarboxylic Acid
16	1% SDS, 2 mM Aurintricarboxylic Acid
17	1% SDS, 1 mM Aurintricarboxylic Acid
18	1% SDS, 0.5 mM Aurintricarboxylic Acid
19	1% SDS, 0.2 mM Aurintricarboxylic Acid
20	1% SDS, 0.1 mM Aurintricarboxylic Acid
21	1% SDS

The tubes were incubated at 55°C with mixing (Eppendorf Thermomixer, Model 5436) for 30 minutes. The resulting surfactant-protease complex was solubilized using methods the same as or similar to those described in Example 7. Undigested tissue fragments were removed by centrifugation using methods the same as or similar to those described in Example 7. The supernatant was phenol extracted, isopropanol precipitated and ethanol washed using methods the same as or similar to those described in Example 6. The washed nucleic acid pellets were resuspended and the integrity of the released nucleic acid were evaluated using methods the same as or similar to those described in Example 6.

As shown in Figure 11, the presence of aurintricarboxylic acid in the reaction composition enhanced the yield of high molecular weight nucleic acid. Reaction compositions containing aurintricarboxylic acid and either CTAB or CTACl released a large amount of high integrity nucleic acid. When SDS was the surfactant in the reaction composition, less nucleic acid was released.

Example 11:

To examine the ability of other cationic reagents to release high integrity nucleic acid, additional compounds were tested as follows. Slices of liver tissue (80 - 140 mg/sample) were placed into fourteen Eppendorf tubes. Five hundred microliters of a reaction composition comprising 100 mM MES, pH 6, 20 mM $CaCl_2$, 0.5 mM aurintricarboxylic acid (e.g., Sigma #A1895), and 1 mg Proteinase K, was added to each tube. One of the cationic reagents shown in Table 11, was added to each tube to a final concentration of 1%. As a control, one tube contained no additional reagent. The tubes were incubated at 60°C for 30 minutes with mixing (Eppendorf Thermomixer,

Model 5436). Following the incubation, 200 µL of a solution containing 1.75 M NaCl, 29% Tween 20, and 585 µg/mL glycogen (resulting in a final concentration of 447 mM NaCl, 7.5% Tween 20, and 149 µg/mL glycogen) was added to each tube to dissolve the surfactant:nucleic acid complex. The remaining tissue fragments were removed by

5 centrifugation using methods the same as or similar to those described in Example 7. The supernatant was phenol extracted, isopropanol precipitated, and ethanol washed using methods the same as or similar to those described in Example 6. The washed nucleic acid pellets were resuspended in 100 µL 10 mM Tris, pH 8.0, 0.1 mM EDTA, and 1 unit/µL Rnasin. The amount of nucleic acid released and its integrity were

10 evaluated using methods the same as or similar to those described in Example 6.

Table 11.

Tube	Cationic Reagent
1	dodecyltrimethylammonium bromide (DTAB)
2	tetradecyltrimethylammonium bromide (TTAB)
3	CTAB
4	CTACl
5, 6	HTAB
7	Mackernium 006 (polyquaternium 6)
8	Mackernium (Olealkonium chloride)
9	Mackernium NLE (Quaternium-84)
10	Mackernium 007 (Polyquaternium 7)
11	Mackernium Stearalkonium SDC85 Chloride
12	Benzalkonium chloride
13	SDS

As shown in Figure 12, all of the cationic reagents tested, except for Mackernium 006 and 007, released high integrity nucleic acid. Of the eleven cationic reagents tested, nine were surfactants and two, Mackernium 006 and 007, were cationic

15 polymers. This result demonstrates that, under these conditions, all of the cationic

surfactants when used in conjunction with Proteinase K, effectively released nucleic acid from the sample.

5   While the present invention has been described in terms of these exemplary embodiments, the skilled artisan will readily understand that numerous variations and modifications of these exemplary embodiments are possible without undue experimentation. All such variations and modifications are within the intended scope of the claimed invention.